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# A Differential Molecular Biology Search for Genes Preferentially Expressed in Functional T Lymphocytes: The CTLA Genes

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## INTRODUCTION

Which molecules are involved in the mechanism of cytotoxicity mediated by cytotoxic T cells (CTLs)? Experimental approaches to answering this question may be classified, according to their working postulates, into functional ones and phenotypic ones.

Some of the *functional studies* aim at inhibiting the cytolytic phenomenology through the blocking of a given CTL molecule. Such inhibition studies with monoclonal antibodies have so far mainly allowed the characterization of molecules mediating specific and non-specific components of CTL-target cell recognition; they have, therefore, brought few insights into the lytic machinery itself that is responsible for the post-recognition "lethal hit" stage.

Other functional studies aim, symmetrically, at reproducing the cytolytic phenomenology using a given molecule purified from CTLs. Thus, subcellular and biochemical fractionations of CTLs and assay of the fractions for their lytic potential have led to the isolation of Perforin (Podack et al. 1985, Masson & Tschopp 1985) (also called Cytolysin (Henkart 1984) and Pore Forming Protein (Young et al. 1986b)); this molecule presents several interesting structural and functional similarities with C9 and is capable, on its own, of killing an array of cells in the presence of extracellular calcium ions. The picture emerging from such studies has recently been complicated, however, by reports of undetectability of perforin in certain types of genuine CTLs (Berke & Rosen 1987, Dennert et al. 1987); by the realization that in some circumstances T cell-mediated cytolysis, which usually requires extracellular calcium, can occur in its absence, whether

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the effector cells are T cell populations (MacLennan et al. 1980, Tirosh & Berke 1985) or clones (Ostergaard et al. 1987, Trenn et al. 1987); by the preliminary purification of at least one additional toxic molecule from CTLs (Liu et al. 1987); and by indications of the occurrence of target cell "induced suicide" pathways (Russell 1983, Ucker 1987). This recent wealth of apparently non-convergent results may well soon crystallize into a new level of integration, including probably more than one mechanism of lysis, for which the molecules identified to date may not entirely account.

The *phenotypic studies* are based on the (sole) assumption that at least some of the molecules involved in CTL-mediated lysis are characteristic of the CTL phenotype, as opposed to any other cellular phenotype. Molecules involved in CTL-mediated lysis might thus be found by looking for molecules specifically expressed in CTLs. This approach amounts to a shift from a functional question to a differentiation question; it renounces the direct phenomenological relevance of functional studies, but may complement them by bypassing some of their intrinsic limitations; it has the advantage of not requiring any preconceived idea as to which molecules might be involved in function and how, but it has the drawback of requiring a subsequent demonstration of functional relevance.

In practice, a search for CTL-specific molecules now often takes advantage of the power of molecular biology techniques, more precisely of the cloning and differential screening of cDNA libraries, both eventually optimized by subtractive enrichment (Alt et al. 1978, 1979, Davis 1986). A number of groups (Lobe et al. 1986, Gershenfeld & Weissman 1986, Koyama et al. 1987, Kwon et al. 1987) including ours (Brunet et al. 1986, 1987b) have used these techniques to try to isolate CTL-specific molecules or, more generally, molecules expressed in lymphocytes (Davis et al. 1984, Hedrick et al. 1984, Kavathas et al. 1984, Saito et al. 1984, Sims et al. 1984, Yanagi et al. 1985, Sakaguchi et al. 1986, Alonso & Weissman 1987, Jongstra et al. 1987). We wish in this report to describe and discuss this approach, as we experienced it, both in terms of molecular biology methodology and in terms of the nature and functional significance of the molecules thus characterized.

## CONSTRUCTION AND SCREENING OF A (CTL-B) cDNA LIBRARY

### *Elements of background*

To detect molecules (in fact the corresponding mRNA transcripts) differentially expressed in cell type A and not in cell type B, a most powerful method is (1) to prepare a *subtracted cDNA library*, by cloning cell type A sequences enriched by subtractive hybridization with cell type B sequences, and (2) to carry out *differential screenings* of this library, with type A, type B, and also type N... cDNA probes.

Subtractive hybridization has been used recently in two distinct ways. First, it was used to enrich a probe or a library for a given, already identified mRNA, using cells known to express or not to express this mRNA (Alt et al. 1978, 1979, Mather et al. 1981); related are the approaches leading to the cloning of TcR$\beta$ (Hedrick et al. 1984) and of CD8 (Kavathas et al. 1984). Second, and directly relevant to the present work, it was used to try to gain access to molecular differences which were not *a priori* known (Zimmerman et al. 1980, Davis et al. 1982, Sargent & Dawid 1983, Cohen et al. 1985).

Differential screenings have been carried out in various combinations, for instance using cDNA probes on libraries of genomic DNA (St. John & Davis 1979) or cDNA (Williams & Lloyd 1979, Rowekamp & Firtel 1980, Lasky et al. 1980, Dworkin & Dawid 1980); poly $A^+$ RNA probes on cDNA libraries (Crampton et al. 1980); subtracted cDNA probes on cDNA libraries (Alt et al. 1979, Mather et al. 1981, Davies et al. 1982, Scott et al. 1983), subtracted cDNA libraries (Sargent & Dawid 1983, Hedrick et al. 1984), or genomic DNA libraries (Zimmerman 1980, Davis et al. 1984). Other combinations have been used, involving e.g. both subtracted and autosubtracted probes (Jongstra et al. 1987), competitive screening (Gershenfeld & Weissman 1986), or sandwich hybridization where the vector is used as a signal amplification intermediate (Boll et al. 1986).

As to sensitivity, reconstitution experiments suggest that the abundance threshold of a given sequence in a probe is 0.01% to 0.06%, for its practical usefulness in library screeenings (Zimmerman et al. 1980, Dworkin & Dawid 1980); this threshold might be lowered by a factor of 10 to 100 using sandwich hybridization (Boll et al. 1986). From a more general point of view, the most sensitive technique would be not to screen a given library, but to use clones of this library, one after the other, as probes on Northern blots (Milner & Sutcliffe 1983).

We have used, to detect unknown differential sequences, a subtracted cDNA library differentially probed with unsubtracted or with subtracted probes. This screening was further optimized by extensive Northern blot analysis. The various steps of this approach are described and commented upon in the next Section.

*Steps of cloning and screening*

Schematical flow chart descriptions of cloning and screening are given in Fig. 1 and in Fig. 2, respectively. More technical aspects (cDNA synthesis, subtractive hybridizations, separation of hybrid molecules on hydroxyapatite columns, cloning and screening procedures...) have been described in detail in other reports (Davis 1986, Brunet et al. 1986).

1. Single-stranded cDNA was synthesized from mRNA extracted from CTLs (an anti-class I, $CD8^+$ murine clone called KB5C20, Albert et al. 1982). This

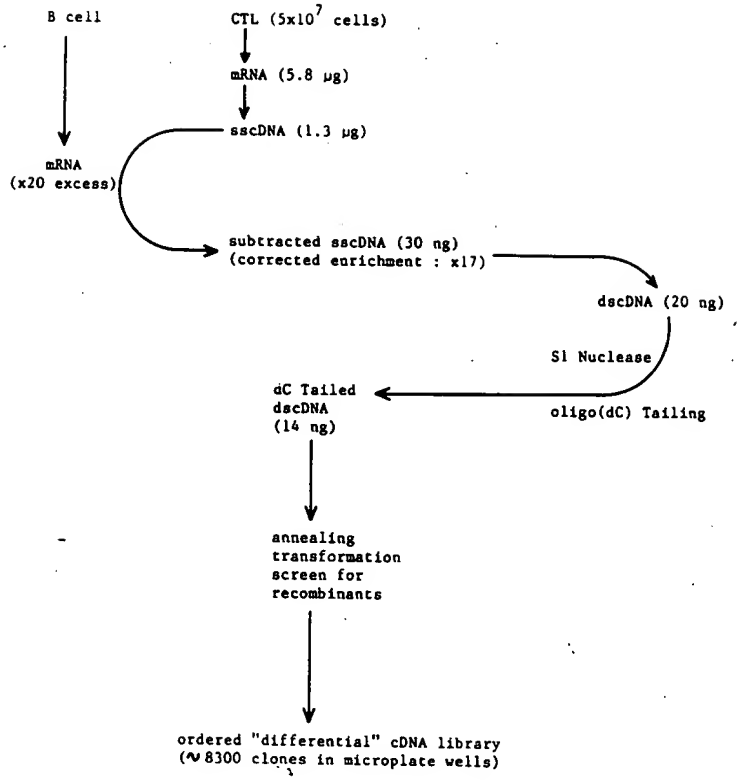



*Figure 1.* Steps in the construction of a given subtracted cDNA library. Single-strand cDNA prepared from the mRNA of a CTL clone (KB5 C20) was subjected twice to hybridization to an excess of mRNA from a B lymphoma (M12-4-1) followed by fractionation on an hydroxyapatite column. The resulting single-strand cDNA was then hybridized to autologous KB5 C20 mRNA. After recovering the double-stand fraction, mRNA was hydrolyzed in alkaline conditions. At this step, the corrected enrichment factor of the recovered single-strand cDNA was of about 17. After synthesis of the second strand, elongation treatment by S1 nuclease and tailing with oligo dC the material was annealed to dG tailed pBR322 vector, then used to transform E. coli (C600). The library included about 8300 recombinants clones. Figures in brackets indicate the amount of recovered material at various steps of the procedure.

cDNA was hybridized twice to an excess of mRNA from B lymphocytes (a B lymphoma called M12.4.1, Hamano et al. 1982). The non-hybridized fraction, enriched in (CTL-B) sequences but also in ill-defined but well-documented (Flint 1980) "non-hybridizable" sequences, is depleted of the latter by hybridization to an excess of autologous mRNA ("positive" hybridization). The initial choice of the subtraction gate conditions the degree of enrichment of the library. The (CTL-

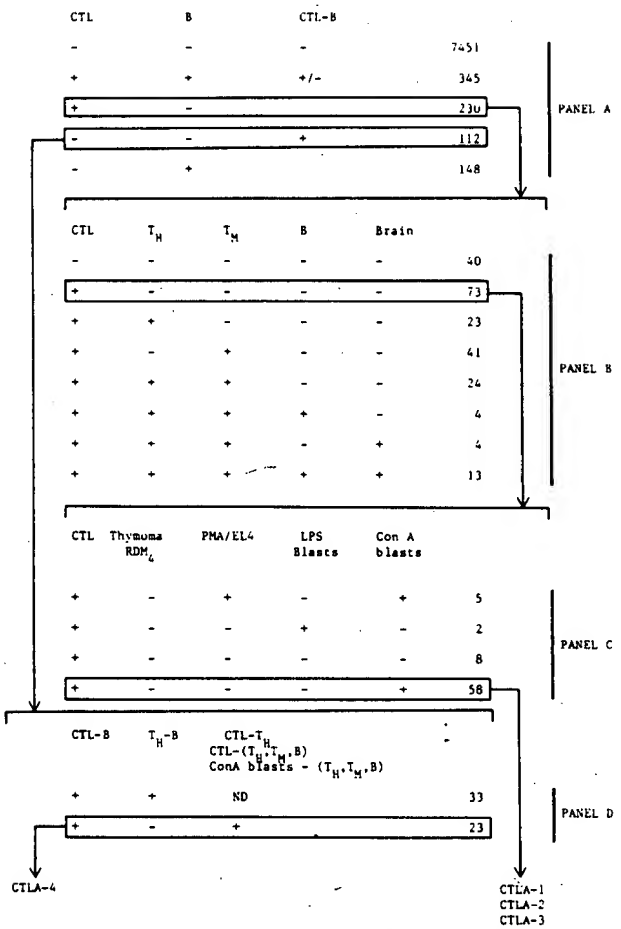



*Figure 2.* Summary of the results obtained after differential screening of the subtracted cDNA library. A first screen of the 8300 clones was done using CTL, B and (CTL-B) radiolabelled cDNA probes (Panel A). The clones positive only with CTL or (CTL-B) probes were subjected to two different rounds of conventional screening (Panel B and C) or to a screening with subtracted probes (Panel D), respectively. In the former case the 58 clones found to be positive only with probes from cytotoxic populations were subjected to cross-hybridization, leading to the characterization of CTL-1, 2 and 3. In the latter case 23 clones were found to hybridize only with CTL cDNA probes subtracted with mRNA from various cell populations. After depletion of clones belonging to the CTLA-1, 2 and 3 families and check of specificity by Northern blots, only one clone was left, called CTLA-4. In the panels above, the figures represent the number of clones giving the indicated pattern with the corresponding cDNA probes (from CTL, a cytotoxic T cell clone; B, a B cell lymphoma; TH, the EL4 thymoma; TM, thymocytes; Brain, mouse brain cells; PMA/EL4, EL4 cells activated with PMA; LPS or Con A blasts, mouse spleen cells activated with lipopolysaccharide or concanavalin A, respectively).

B) gate is a compromise aiming at both reasonable pertinence and abundance of cDNA after subtraction. A narrower gate, (CTL-TH), was used by others (Kwon et al. 1987) with success.

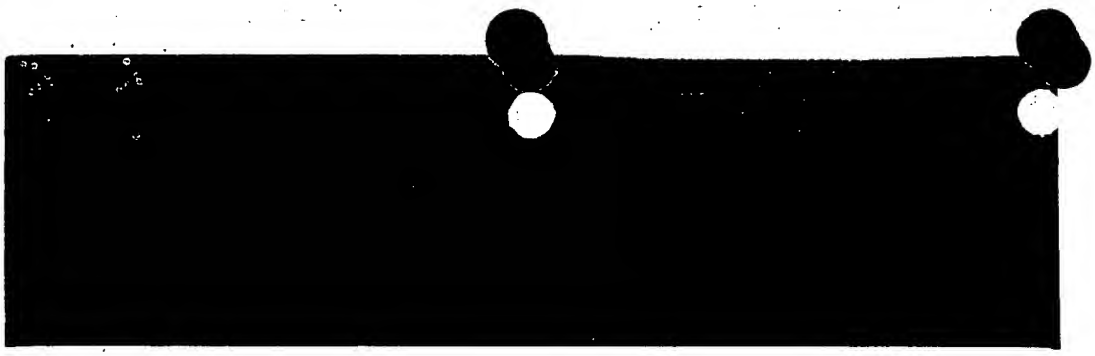
2. The factor of enrichment of cDNA in sequences differentially expressed between CTL and B, computed from hybridization data, was in our case a factor of 17. Such an arithmetical result should be considered with caution. We noticed, upon screening, inconsistencies between the degree of hybridization to certain clones (which reflects the abundance of the corresponding mRNA) and their redundancy within the library; this suggests that some bias may occur for certain sequences either at the cloning or at the subtraction stage.

3. The subtracted cDNA was processed as described in Fig. 1. The few nanograms of resulting material were used to construct a library of about 8300 clones which were subsequently individually distributed in microplates. These could be stored and used at will to prepare filters bearing clones in ordered patterns. Such a permanent and ordered collection of recombinant plasmid clones (Gergen et al. 1979) is especially adequate for a relatively small number of clones, thus for a subtracted library; it allows, through multiple differential screenings, cumulative records to be kept on the characteristics of individual clones.

4. Using filter replicas bearing ordered patterns of clones, the 8300 clones were subjected to successive rounds of differential screenings (Fig. 2) with cDNA probes representing mRNA populations from a range of CTL and non-CTL cells. The rationale of these successive positive and negative rounds of screening was to dissociate, as far as possible, molecules correlating with the T-type lytic phenomenology from those only phenotypically linked to it but actually irrelevant, related for instance to the "T-ness" of CTLs, or to their activated state, or to clonal idiosyncrasies, or to *in vitro* aberrations. Only 58 clones survived these screenings, in that they hybridized with cDNA probes from CTL and did not hybridize with probes from non-CTL. The yield of these successive rounds of screening, in terms of progressive depletion of the library, decreased with each round to a point where further rounds would have been of very limited usefulness.

5. The 58 $CTL^+$ non-$CTL^-$ clones were brought down, by systematic cross-hybridizations and mRNA sizing, to 3 clearly different cDNAs, defining CTLA-1, CTLA-2 and CTLA-3.

6. In parallel, the initial subtracted library was screened again, this time with a subtracted (CTL-B) cDNA probe. The $CTL^-$, $B^-$, $(CTL\text{-}B)^+$ clones thus detected were subjected to further screenings with subtracted (CTL-non CTL) probes, leading to the isolation of 23 $(CTL\text{-non CTL})^+$ clones. Finally, it was of interest that only a total of about 300 clones (4%) out of the 8300 clones of this CTL library gave a CTL-specific signal; ⅔ of these 300 were detected with a non-subtracted CTL cDNA probe, and ⅓ required a subtracted (CTL-B) cDNA probe for detection. Also, about 150 clones were $CTL^-B^+$, which we interpret as





corresponding to transcripts expressed more in B cells than in CTL, but not much more since otherwise they would have been subtracted away.

7. The 23 $CTL^-$, $(CTL\text{-}non\ CTL)^+$ clones were depleted, by specific screenings, of a number of CTLA-1 to CTLA-3 clones. That these had not been detected through the initial direct screening with CTL probes shows some of the limits of reliability of this approach; an explanation might be found in the relative position of partial clones compared to the mRNA, combined to the 3′ bias of oligo-dT-initiated cDNA probes.

8. The tissue specificity of the selected clones was checked by Northern blots, amounting to a reverse and more sensitive screening. This led us first – using cells already used in direct screenings – to check and in places to disprove the "CTL specificity" of some clones, especially those detected by subtracted probes, of which only one was finally retained, defining CTLA-4. The sensitivity of direct screening (*in situ* hybridization of clones and cDNA probes) is predictably less than that (almost symmetrical) of Northern blots; results obtained with the latter led us to significantly qualify the results obtained with the former. The gap between both sets of results increases for low-abundance mRNAs; for these, direct screening tends to become ineffective.

9. We then extended this Northern blot assessment of tissue distribution to many other cell types: other cytolytic clones or hybridomas, *in vitro* and *in vivo* generated cytolytic populations of the T and NK type, non-cytolytic T cells, B cells, several lines of macrophages and mast cells, plus fibroblasts, liver, brain, adrenals and a neurosecretory line. It should be noted that this list contains a significant gap: as a consequence of a phenomenological (and unavoidable) purism as to the definition of "cytolytic" and "non-cytolytic" cell types (any leakage to cytotoxic activity, which we very often observed with cells considered to be TH, rigorously preventing any conclusion) we could not test unquestionably "non-cytolytic" activated T cells (apart from PMA-activated EL4); we were thus not able at this stage to dissociate the cytolytic T phenotype from, more generally, the mature (functional) activated T phenotype. This potential confusion apart, the picture of the tissue distribution of CTLA-1 to CTLA-4 transcripts, summarized in Table I, is clearly centered on the cytolytic T cell type with a number of leakings – especially in non cytolytic T cells (like thymocytes) – and two massive exceptions: a strong expression of CTLA-2 in one macrophage line and of CTLA-1 and CTLA-2 in all mast cell types tested (except for one tumor).

In addition to the relative overall similarity as to tissue expression of the various CTLAs, it was found (Brunet et al. 1986, 1987b) that transcription of all the CTLA genes was inducible under the same lymphocyte activation conditions. This raises interesting questions as to possible homologies of control elements for the various CTLA genes.

We are now going to briefly describe the structure of the CTLA-1 to CTLA-4 transcripts, which all happen to have significant homology to known mol-

TABLE I

*A summary of tissue-specificity patterns of CTLA-1 to CTLA-4 transcripts*

| | Cyto-toxicity | CTLA-1 | CTLA-2 | CTLA-3 | CTLA-4 |
|---|---|---|---|---|---|
| Cytotoxic T cell populations | | | | | |
| Primary MLC | + | +++ | + | + | ++ |
| Con A blasts | + | +++ | + | + | +++ |
| Thymocytes + Con A + SN | + | +++ | ++ | ++ | ++ |
| Peritoneal Exudate Lymphocytes | + | +++ | +++ | + | ++ |
| Cytotoxic T clones | | | | | |
| KB5 C20 | + | +++ | +++ | +++ | ++ |
| A15 1 17 | + | +++ | ++ | ± | + |
| T hybridomas | | | | | |
| Induced PC60 | + | +++ | + | +++ | ++ |
| SPH | ± | ± | + | ++ | − |
| MN2 | + | + | + | +++ | ± |
| FLH-S | + | + | ++ | ++ | − |
| NK cell populations | | | | | |
| Fractionated nude (nu/nu) spleen cells | + | + | + | + | ± |
| Non-cytotoxic T cells | | | | | |
| Thymocytes | − | − | ± | ± | ± |
| Thymocytes + Con A | − | − | ± | ± | ± |
| EL4 | − | − | − | − | − |
| T14.117 (helper hybridoma) | − | − | ± | − | − |
| Uninduced PC60 | − | − | − | + | + |
| B lymphocytes | | | | | |
| LPS blasts | − | − | ± | ± | + |
| M12.4.1 (lymphoma) | − | − | − | − | − |

ecules, thus allowing at least some preliminary speculation as to their biological role.

## CTLA-1 AND CTLA-3

The open reading frames of CTLA-1 (also cloned by others as CCP1 (Lobe et al. 1986)) and CTLA-3 (also cloned as H Factor, (Gershenfeld & Weissman 1986)) of 741 and at least 738 nucleotides, respectively, potentially encode proteins with extensive homology to members of the serine protease family. For instance, CTLA-1 is 46% and 40% similar to Rat Mast Cell group-specific Protease and to the D Factor of human complement, respectively. CTLA-3 (H Factor) has been shown to be 35% similar to Rat Mast Cell group-specific protease.

Parallel studies have led to the detection of these molecules at the protein level, together with at least 6 other very similar ones, all located in CTL granules, and

designated under the generic term of granzymes (Masson & Tschopp 1987). This abundance of distinct proteases has prompted speculation as to their participation in an enzymatic cascade, of which the complement system is an example. There is, indeed, an apparent convergence of these molecular findings and of earlier models (Lachmann 1983) and evidence (Ferluga et al. 1972, Redelman & Hudig 1980) involving serine proteases in cell-mediated cytotoxicity. As far as complement-like models of cytotoxicity are concerned, the serine-protease granzyme A (CTLA-3, H Factor) has the disturbing property of being found in equal amounts in the $CD4^+$ (with low cytolytic ability) and $CD8^+$ (with high cytolytic ability) compartments of activated T cells (Garcia-Sanz et al. 1987). As to inhibition studies: 1) the susceptibility of cell-mediated killing to the serine protease inhibitor TLCK (Matter 1975) is not obviously reflected by that of any of the isolated proteases tested to date (Pasternack & Eisen 1985, Young et al. 1986a), and 2) there might be some involvement in this inhibition phenomenology of membrane-

TABLE I
*Continued*

| | Cyto-toxicity | CTLA-1 | CTLA-2 | CTLA-3 | CTLA-4 |
|---|---|---|---|---|---|
| Macrophage cell lines | | | | | |
| IC21 | − | − | ++ | − | − |
| PU5 | − | − | − | − | − |
| Activated PU5 | − | − | − | − | − |
| P388D1 | − | − | − | − | − |
| Activated P388D1 | + | − | − | ND | ND |
| Mast cells | | | | | |
| P815 (mastocytoma) | +(NC) | − | ++ | − | − |
| Bone marrow derived mast cells | ND | +++ | ++ | ND | − |
| ABFTL1 (mastocytoma) | +(NC) | ++ | +++ | − | ND |
| Others | | | | | |
| L cells (fibroblast line) | − | − | − | − | − |
| AtT.20 (neurosecretory cell line) | − | − | − | − | − |
| Brain cells | − | − | ± | ± | − |
| Liver cells | − | − | − | − | − |
| Adrenal cells | − | − | ND | − | ND |

A summary of specificity patterns of CTLA-1, CTLA-2, CTLA-3 and CTLA-4 transcripts. Northern blots of RNA from the indicated cells or tissues were probed with the inserts of plasmids representative of the different CTLAs. The symbols − to +++ indicate from undetectable to high levels of the corresponding transcript in the various cell types tested. Cells were also tested for cytotoxic activity in a conventional $^{51}Cr$ release assay using EL4 or YAC as target cells in the presence of Concanavalin A (10 μg/ml). For natural cytotoxicity (NC) determination, a 20 hours $^{51}Cr$ assay using WEH1 164 as target cells was used. ND: not determined. The data are from Brunet et al. 1986, 1987a, 1987b and from Denizot et al. in preparation.

bound enzymes at the level of early transmembrane signalling (Utsunomiya & Nakanishi 1986). All these arguments, however, do not exclude a possible role for CTLA-1 or CTLA-3 or other granzymes in at least some types of T cell-mediated cytolysis.

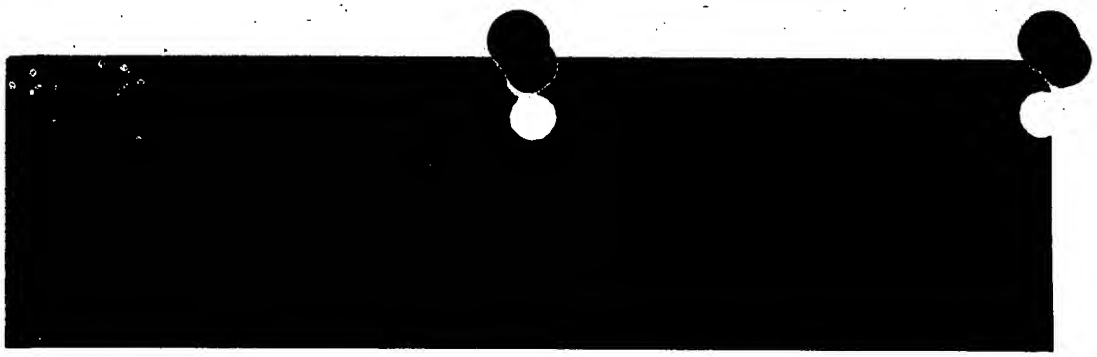
Apart from these functional speculations, the presence of CTLA-1 and CTLA-2 – or even, in the hypothesis of cross hybridization, of very similar enzymes – in activated T cells and mast cells (Brunet et al. 1987a) must be noted as a phenotypic likeness between these two cell types, in line with their still speculative ontogenic relationship.

## CTLA-2

Two different cDNAs (CTLA-2$\alpha$ and CTLA-2$\beta$) were characterized. They are 680 and 777 nucleotides long, respectively, and are 95% identical in their 5′ end regions on more than 630 nucleotides. The open reading frames are 414 and 423 nucleotides long, respectively, but the starting ATG codon is known only in the case of CTLA-2$\alpha$. The corresponding protein sequences, which are more than 90% identical, both have significant homology to the pro-region of the mouse cysteine proteinase MCP. They also share a number of constant residues with 3 other pro-regions of cysteine proteinases from different species. Thus, CTLA-2$\alpha$ and $\beta$ seem to be intracellular autonomous homologues of cysteine proteinase pro-regions, preferentially expressed in activated T lymphocytes and mast cells (F. Denizot et al., in preparation). The functional significance of these molecules remains to be ascertained; a protease-inhibiting activity, however, is not unlikely.

## CTLA-4

The protein sequence of the 669 nucleotide-long open reading frame of CTLA-4 revealed primary structure features characteristic of members of the Ig superfamily, clustered around two cysteine residues (58 and 129) positionally homologous to the two canonical Cys of V domains (Brunet et al. 1987b). Interpreted in this light, CTLA-4 appears as a V-like domain, with a most probable anchor at the cell surface, accounting for the hydrophobic stretch 3′ of the V domain, followed by a putative cytoplasmic domain. CTLA-4 is an addition to the exponentially growing subfamily of membrane-bound single V domains, already comprising Thy 1 (Campbell et al. 1979), 3 chains of the CD3 complex (Gold et al. 1986, Van den Elsen et al. 1984), the 2 chains of CD8 (Kavathas et al. 1984, Nakauchi et al. 1985), and more recently CD7 (Aruffo & Seed 1987a) and the homodimer CD28 (Aruffo & Seed 1987b). Although different from CD28, CTLA-4 shares with it some significant structural features, such as the precise number and relative position of cysteines, and a proline-rich stretch near the hydrophobic portion. It may be noted that a number of these molecules are involved in





transmembrane signalling. That this might also be the case for CTLA-4 is certainly worth investigating, for instance through the use of antibodies. In line with this possibility is the fact that, in the cytoplasmic region of the molecule, 100% of amino acid residues are conserved between the human and murine homologues of CTLA-4 (P. Dariavach, M. P. Lefranc et al., in preparation). Also the antibody approach could help in refining the distribution of CTLA-4 among T cell populations (including thymocytes for which a faint signal is seen in Northern blots) and detecting any involvement in CTL/target adhesion or more generally in T cell functions.

## DISCUSSION

The differential molecular phenotyping approach which we and others used, and the postulate of specificity on which it was founded, are not devoid of risks and limitations. For instance, it is conceivable that non-CTL-specific molecules may participate in CTL-specific functions; such molecules might be expressed, for instance, not only in CTL but also in non-cytolytic CTL precursors (like thymocytes); these molecules would of course be lost in the course of a differential search and the more thorough this search, the more surely would these molecules be lost. Another possibility is that the phenomenological correlate of this phenotype approach (in our case a 4-h $^{51}Cr$ release assay) may not distinguish between different types of CTLs, exerting cytolysis via different mechanisms; in this case, a phenotypic correlation with only a given subtype of CTL-mediated cytotoxicity could be wrongly interpreted as an indication of irrelevance to CTL-mediated cytotoxicity as a whole. Bearing these limitations in mind, but given that they are different from those of the "functional" studies, one can hope that this approach will help to add some interesting pieces to the fast-developing molecular puzzle of T cell functions.

The transcripts corresponding to the 5 molecules we cloned have a limited spectrum of tissue distribution, centered on the cytolytic T phenotype, and probably more generally on the activated mature T phenotype. The latter point has been shown by others for CTLA-3 (Garcia Sanz et al. 1987) and remains to be assessed (probably optimally using antibodies) for CTLA-2 and CTLA-4. It should be noted that the specificity criterion which led to this approach had to be revised in the course of the search, none of the CTLAs being strictly CTL-specific or even strictly T-specific (which does not argue definitively against their involvement in T lymphocyte functions). Considering the initial specificity postulate, this adds a factual limitation to the theoretical ones mentioned above. Also, this probably limits, in retrospect, the legitimacy of some of the screening steps. More generally, no molecule to date can be claimed to be strictly CTL-specific.

Beyond the speculations allowed by structural similarities, one can already

begin to ask questions about the functional aspects of the molecules we and others have isolated using differential molecular biology techniques. This may be achieved through various strategies. The first one consists, after having conceptually jumped from function to genes (but through a phenotypic correlation), in going back to (recombinant) proteins and raising antibodies against them. This should allow us at least to refine descriptive aspects of detailed cellular distribution and subcellular localization, and perhaps for surface-located molecules to look at the blocking or triggering of testable functions. The second strategy consists in jumping back from genes to function, through unambiguous causal links. This may include the derivation of cell variants having lost the cytolytic function and, simultaneously, one or several CTLA genes. These variants could provide a cellular material lending itself, through transfection, to functional rescue. Another possibility is to try and block testable functions through the use of antisense nucleic acids. All these approaches are strictly dependent on the variety of phenomenological readouts available to monitor T cell functions.

Yet, already, and even before the phenomenological and reverse genetics progress that this kind of approach is clearly calling for, CTLA-1 to 4 can be added to the ever growing molecular picture of the mature T lymphocyte phenotype. In no other system have so many differential clonings been undertaken, so that the "functional T phenotype" is on the way to becoming one of the best explored, at least in these strictly descriptive terms. The likely progressive convergence of these studies, and saturation of the molecular field reachable by these and other means, far from being a matter of strategical concern for those in it or about to enter it, should be an encouragement to go on. Indeed, pushing these horizontal explorations of the T phenotype(s) could mean gaining at least preliminary insights into a complete definition, but now at the molecular level, of the T cell compartment, which is certainly relevant to the ultimate understanding of T cell functions.

## SUMMARY

One approach to the isolation of molecules involved in T cell-mediated cytolysis stems from the postulate of a possible correlation between molecular phenotype and molecular functional involvement. Accordingly, CTL-specific molecules have been looked for, using a strategy based on the differential screening of a subtracted cDNA library. This led to the isolation and characterization of the following structures, expressed mostly (but no exclusively) in CTLs and inducible upon lymphocyte activation: CTLA-1 and CTLA-3 (serine-proteases), CTLA-4 (a member of the Ig superfamily) and CTLA-2$\alpha$ and $\beta$ (homologues to the proregion of cysteine-proteases). The theoretical and practical limitations and the prospects of this type of approach are discussed.

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## REFERENCES

Alonso, M. A. & Weissman, S. M. (1987) cDNA cloning and sequence of MAL, a hydrophobic protein associated with human T-cell differentiation. *Proc. Natl. Acad. Sci. USA* **84**, 1997.

Albert, F., Buferne, M., Boyer, C. & Schmitt-Verhulst, A. M. (1982) Interactions between MHC-encoded products and cloned T cells 1. Fine specificity for induction of proliferation and lysis. *Immunogenetics* **16**, 533.

Alt, F. W., Enea, V., Bothwell, A. L. M. & Baltimore, D. (1979) Probes for specific mRNAs by subtractive hybridization: anomalous expression of immunoglobulin genes. In: *Eucaryotic Gene Regulation, UCLA symposia on Molecular and Cellular Biology*. Academic Press, 407.

Alt, F. W., Kellems, R. E., Bertino, J. R. & Schimke, R. T. (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate – resistant variants of cultured murine cells. *J. Biol. Chem.* **253**, 1357.

Aruffo, A. & Seed, B. (1987a) Molecular cloning of two CD7 (T cell leukemia antigen) cDNAs by a COS cell expression system. *EMBO J.* **6**, 3313.

Aruffo, A. & Seed, B. (1987b) Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA* **84**, 8573.

Berke, G. & Rosen, D. (1987) Are lytic granules and Perforin 1 involved in lysis induced by in vivo-primed peritoneal exudate cytolytic T lymphocytes? *Transplant. Proc. XIX*, 412.

Boll, W., Fujisawa, J. I., Niemi, J. & Weissmann, C. H. (1986) A new approach to high sensitivity differential hybridization. *Gene* **50**, 41.

Brunet, J. F., Denizot, F., Suzan, M., Haas, W., Mencia Huerta, J. M., Berke, G., Luciani, M. F. & Golstein, P. (1987a) CTLA-1 and CTLA-3 serine esterase transcripts are detected mostly in cytotoxic T cells, but not only and not always. *J. Immunol.* **138**, 4102.

Brunet, J. F., Denizot, F., Luciani, M.-F., Roux-Dosseto, M., Suzan, M., Mattei, M. G. & Golstein, P. (1987b) A new member of the immunoglobulin superfamily – CTLA4. *Nature* **328**, 267.

Brunet, J. F., Dosseto, M., Denizot, F., Mattei, M. G., Clark, W. R., Haqqi, T. M., Ferrier, P., Nabholz, M., Schmitt-Verhulst, A. M., Luciani, M. F. & Golstein, P. (1986) The inducible cytotoxic T-lymphocyte associated gene transcript CTLA-1 sequence and gene localization to mouse chromosome 14. *Nature* **322**, 268.

Campbell, D. G., Williams, A. F., Bayley, P. M. & Reid, K. B. M. (1979) Structural similarities between Thy 1 antigen from rat brain and immunoglobulins. *Nature* **282**, 341.

Cohen, D. I., Hedrick, S. M., Nielsen, E. A., D'Eustachio, P., Ruddle, F., Steinberg, A. D., Paul, W. E. & Davis, M. M. (1985) Isolation of a cDNA clone corresponding to

an X-linked gene family (XLR) closely linked to the murine immunode[illegible] xid. *Nature* **314**, 369.

Crampton, J., Humphries, S., Woods, D. & Williamson, R. (1980) The iso[illegible] cDNA sequences which are differentially expressed in human lymph[illegible] blasts. *Nucl. Acids Res.* **8**, 6007.

Davis, M. M. (1986) Subtractive cDNA hybridization and the T-cell rec[illegible] *Cellular Immunology* Vol. 2. Blackwell Scientific Publications.

Davis, M. M., Cohen, D. I., Nielsen, E. A., De Franco, A. L. & Paul, W[illegible] isolation of B and T cell-specific genes in B and T cell tumors. In: *UC[illegible] Molecular and Cellular Biology*, Volume XXIV. Vitteta, E. S., ed. Ac[illegible]

Davis, M. M., Cohen, D. I., Nielsen, E. A., Steinmetz, M., Paul, W. [illegible] (1984) Cell-type-specific cDNA probes and the murine I region: the [illegible] orientation of Aα. *Proc. Natl. Acad. Sci. USA* **81**, 2194.

Dennert, G., Anderson, C. G. & Prochazka, G. (1987) High activity of N[illegible] bonyl-L-lysine thiobenzyl ester serine esterase and cytolytic perforin i[illegible] is not demonstrable in in-vivo-induced cytotoxic effector cells. *Proc[illegible] USA* **84**, 5004.

Dworkin, M. B. & Dawid, I. B. (1980) Use of a cloned library for the st[illegible] poly (A)$^+$ RNA during Xenopus leavis development. *Dev. Biol.* **76**, [illegible]

Ferluga, J., Asherson, G. L. & Becker, E. L. (1972) The effect of or[illegible] inhibitors, p-Nitrophenol and cytochalasin B on cytotoxic killing of [illegible] immune spleen cells, and the effect of shaking. *Immunology* **23**, 577.

Flint, S. J. (1980) Measurement of messenger RNA concentration. In: *Gen[illegible] Principles and Methods*, Vol. 2. Plenum Press.

Garcia-Sanz, J. A., Plaetnick, G., Velotti, F., Masson, D., Tschopp, J., [illegible] R. & Nabholz, M. (1987) Perforin is present only in normal ac[illegible] lymphocytes and not in L3T4$^-$ cells, but the serine protease granzym[illegible] both subsets. *EMBO J.* **6**, 933.

Gergen, P., Stern, R. H. & Wensink, P. C. (1979) Filter replicas and perm[illegible] of recombinant DNA plasmids. *Nucl. Acids Res.* **7**, 2115.

Gershenfeld, H. K. & Weissman, I. L. (1986) Cloning of a cDNA for a T c[illegible] protease from a cytotoxic T lymphocyte. *Science* **232**, *854*.

Gold, D. P., Puck, J. M., Petty, C. L., Cho, M., Coligan, J., Woody, J. N[illegible] (1986) Isolation of cDNA clones encoding the 20 K non-glycosyl[illegible] chain of the human T-cell receptor/T3 complex. *Nature* **321**, 431.

Hamano, T., Kim, K. J., Leiserson, W. M. & Asofsky, R. (1982) Establi[illegible] hybridomas with B cell surface antigens. *J. Immunol.* **129**, 1403.

Hedrick, S. M., Cohen, D. I., Nielsen, E. A. & Davis, M. M. (1984) Iso[illegible] clones encoding T cell-specific membrane-associated proteins. *Natur[illegible]*

Henkart, A. P., Millard, P. J., Reynolds, C. W. & Henkart, M. P. (1984) [illegible] of purified cytoplasmic granules from cytotoxic rat large granular ly[illegible] *J. Exp. Med.* **160**, 75.

Jongstra, J., Schall, T. J., Dyer, B. J., Clayberger, C., Jorgensen, J., L[illegible] Krensky, A. M. (1987) The isolation and sequence of a novel gen[illegible] functional T cell line. *J. Exp. Med.* **165**, 601.

Kavathas, P., Sukhatme, V. P., Herzenberg, L. A. & Parnes, J. R. (1984[illegible] gene encoding the human T-lymphocyte differentiation antigen Le[illegible] transfer and cDNA subtraction. *Proc. Natl. Acad. Sci. USA* **81**, 768.

Koyama, T., Hall, L. R., Haser, W. G., Tonegawa, S. & Saito, H. (198[illegible] cytotoxic T-lymphocyte-specific gene shows a strong homology to fi[illegible] chains. *Proc. Natl. Acad. Sci. USA* **84**, 1609.

Kwon, B. S., Kim, G. S., Prystowsky, M. B., Lancki, D. W., Sabath, D. E., Pan, J. & Weissman, S. M. (1987) Isolation and initial characterization of multiple species of T-lymphocyte subset cDNA clones. *Proc. Natl. Acad. Sci. USA* **84**, 2896.

Lachmann, P. J. (1983) Are complement lysis and lymphocytotoxicity analogous? *Nature* **305**, 473.

Lasky, L. A., Lev, Z., Xin, J. H., Britten, R. J. & Davidson, E. H. (1980) Messenger RNA prevalence in sea urchin embryos measured with cloned cDNAs. *Proc. Natl. Acad. Sci. USA* **77**, 5317.

Liu, C.-C., Steffen, M., King, F. & Young, J. D.-E. (1987) Identification, isolation and characterization of a novel cytotoxin in murine cytolytic lymphocytes. *Cell* **51**, 393.

Lobe, C. G., Havele, C. & Bleackley, R. C. (1986). Cloning of two genes that are specifically expressed in activated cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **83**, 1448.

MacLennan, I. C. M., Gotch, F. M. & Golstein, P. (1980) Limited specific T-cell mediated cytolysis in the absence of extracellular $Ca^{2+}$. *Immunology* **39**, 109.

Masson, D. & Tschopp, J. (1987) A family of serine-esterases in lytic granules of cytolytic T lymphocytes. *Cell* **49**, 679.

Mather, E. L., Alt, F. W., Bothwell, A. L. M., Baltimore, D. & Koshland, M. E. (1981) Expression of J Chain RNA in cell lines representing different stages of B lymphocyte differentiation. *Cell* **23**, 369.

Matter, A. (1975) A study of proteolysis as a possible mechanism for T-cell mediated target cell lysis. *Scand. J. Immunol.* **4**, 349.

Nakauchi, H., Nolan, G. P., Hsu, C., Huang, H. S., Kavathas, P. & Herzenberg, L. A. (1985) Molecular cloning of Lyt-2, a membrane glycoprotein marking a subset of mouse T lymphocytes: molecular homology to its human counterpart, Leu 2/T8, and to immunoglobulin variable regions. *Proc. Natl. Acad. Sci. USA* **82**, 5126.

Ostergaard, H. L., Kane, K. P., Mescher, M. F. & Clark, W. R. (1987) Cytotoxic T lymphocyte mediated lysis without release of serine esterase. *Nature* **330**, 71.

Pasternack, M. & Eisen, H. (1985) A novel serine esterase expressed by cytotoxic T lymphocytes. *Nature* **314**, 743.

Rowekamp, W. & Firtel, R. A. (1980) Isolation of developmentally regulated genes from Dictyostelium. *Develop. Biol.* **79**, 409.

Russell, J. H. (1983) Internal disintegration model of cytotoxic lymphocyte-induced target damage. *Immunol. Rev.* **72**, 97.

Saito, H., Kranz, D. M., Takagaki, Y., Hayday, A. C., Eisen, H. N. & Tonegawa, S. (1984) Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature* **309**, 757.

Sakaguchi, N., Berger, C. N. & Melchers, F. (1986) Isolation of a cDNA copy of an RNA species expressed in murine pre-B cells. *EMBO J.* **5**, 2139.

Sargent, T. D. & Dawid, I. B. (1983) Differential gene expression in the gastrula of Xenopus laevis. *Science* **222**, 135.

Scott, M. R. D., Westphal, K. H. & Rigby, P. W. J. (1983) Activation of mouse genes in transformed cells. *Cell* **34**, 557.

Sims, J. E., Tunnacliffe, A., Smith, W. J. & Rabbitts, T. H. (1984) Complexity of human T cell antigen receptor $\beta$-chain constant- and variable-region genes. *Nature* **312**, 541.

St. John, T. P. & Davis, R. W. (1979) Isolation of galactose-inducible DNA sequences from saccharomyces cerevisiae by differential plaque filter hybridization. *Cell* **16**, 443.

Tirosh, R. & Berke, G. (1985) T lymphocyte mediated cytolysis as an excitatory process of the target. I. Evidence that the target cell may be the site of $Ca^{++}$ action. *Cell. Immunol.* **95**, 113.

Trenn, G., Takayama, H. & Sitkovsky, M. V. (1987) Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. *Nature* **330**, 72.

Ucker, D. S. (1987) Cytotoxic T lymphocytes and glucocorticoids activate an endogenous suicide process in target cells. *Nature* **327**, 62.

Utsunomiya, N. & Nakanishi, M. (1986) A serine protease triggers the initial step of transmembrane signalling in cytotoxic T cells. *J. Biol. Chem.* **261**, 16514.

Van Den Elsen, P., Shepley, B. A., Borst, J., Coligan, J. E., Markham, A. F., Orkin, S. & Terhorst, C. (1984) Isolation of cDNA clones encoding the 20K T3 glycoprotein of human T-cell receptor complex. *Nature* **312**, 413.

Williams, J. G. & Lloyd, M. M. (1979) Changes in the abundance of polyadenylated RNA during slime mould development measured using cloned molecular hybridization probes. *J. Mol. Biol.* **129**, 19.

Yanagi, Y., Chan, A., Chin, B., Minden, M. & Mak, T. W. (1985) Analysis of cDNA clones specific for human T cells and the $\alpha$ and $\beta$ chains of the T-cell receptor heterodimer from a human T-cell line. *Proc. Natl. Acad. Sci. USA* **82**, 3430.

Young, J. D.-E., Leong, L. G., Liu, C. C., Damiano, A., Wall, D. A. & Cohn, Z. A. (1986a) Isolation and characterization of a serine esterase from cytolytic T-cell granules. *Cell* **47**, 183.

Young, J. D.-E., Podack, E. R. & Cohn, Z. A. (1986b) Properties of a purified pore-forming protein (Perforin 1) isolated from H-2-restricted cytotoxic T cell granules. *J. Exp. Med.* **164**, 144.

Zimmerman, C. R., Orr, W. C., Leclerc, R. F., Barnard, E. C. & Timberlake, W. E. (1980) Molecular cloning and selection of genes regulated in Aspergillus development. *Cell* **21**, 709.